PPG Industries, Inc. 4325 Rosanna Drive Allison Park, Pennsylvania 15101
Phone: 412 492-5308 FAX: 412 492-5611 Email: sabraham@ppg.com

CONTAINS NO CBI

March 17, 2005

8EHQ-0305-15971

DHL 758 2325 310

Document Processing Center (7407M)
EPA East – Room 6428
Attn: Section 8(e)
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

To Whom It May Concern:

PPG Industries Inc., (PPG) is submitting this information pursuant to Section 8(e) of TSCA.

PPG is submitting reports on two aquatic toxicity studies concerning WRS-2390, CAS# 68227-46-3, an isolated intermediate chemically converted in subsequent production of a resin that is a component of an electrodeposition coating. As a part of EPA's high production volume challenge program, toxicity studies were conducted on WRS-2390.

Enclosed you will find the full reports, including summaries, titled:

- Determination of Acute Toxicity (EC50) to Daphnia (48h, static)
- Alga, Growth Inhibition Test (96h, EC50)

PPG provides our associates with labels and MSDS, which specify procedures for proper handling and disposal of this material including the use of personal protective equipment.

Please telephone me at [412 492-5308] if you have any questions.

Sincerely yours,

Stacie Abraham
Senior Product Safety Analyst
North America and European Regions
PPG Industries, Inc.

attachments

284536

CONFIDENTIAL

Inveresk Report Number 24139

WRS-2390TX
Determination of Acute Toxicity (EC_{50}) to Daphnia (48 h, Static)

Authors:

T L Hargreaves
C R Kelly
C M Murphy

Sponsor:

PPG Industries Inc
4325 Rosanna Drive
P O Box 9
Allison Park
Pennsylvania 15101
USA

Performing Laboratory:

Inveresk
Tranent
EH33 2NE
Scotland

Final Page of Report: 26

Contents

Authentication

'I, the undersigned, hereby declare that this work was performed in accordance with the OECD Principles of Good Laboratory Practice as set forth by the United Kingdom Department of Health. The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained.'

T. Hargreaves

T L Hargreaves BSc
Study Director

14 January 2005
Date

Quality Assurance Statement

The execution of this type of short-term study is not individually inspected. The processes involved are inspected by the Inveresk Quality Assurance Unit at intervals according to a predetermined schedule.

Date of QA Inspection	Phase	Date of Report to Management
24 March 2004	Addition of Test Item / Addition of Test Organisms / Preparation of Vessels / Water Quality Parameters	25 March 2004
06 December 2002	Protocol Review	10 December 2002

The protocol review was also reported to the Study Director.

The report has been audited by the Quality Assurance Personnel according to the appropriate Standard Operating Procedure(s). The report is considered to describe accurately the methods and procedures used in the study. The reported results accurately reflect the original data generated during the study.

D. Allan
D Allan BSc
Quality Assurance

12 Jan 05
Date

Personnel Involved

Study Director:	T L Hargreaves BSc (28 July 2004 – Study Completion)
	C R Kelly BSc PhD (30 September 2003 – 27 July 2004)
	B Knight BSc (02 December 2002 – 29 September 2003)
Head, Environmental Sciences:	B E Hall BSc PhD
Report Compilation:	T L Hargreaves BSc C R Kelly BSc PhD C M Murphy BSc
Scientific Assistance:	C M Murphy BSc J M Hughes BSc PhD
Sample Analysis:	A Hogg BSc W Brown BSc
Quality Assurance:	D Allan BSc K McDonald BSc

1 Summary

The median effective concentration (EC_{50}) for immobilisation of *Daphnia magna* exposed to WRS-2390TX was determined over a 48 h period. The study was conducted under static conditions in accordance with OECD (1984) Guideline 202 Part I and EC (2001) Guideline C2.

Based on the results of range finding tests, a definitive test was conducted with twenty *Daphnia* at each treatment group and control, as 4 replicates of 5, at 0.64, 1.4, 3.0, 6.4, and 14 mg/L total test item with an untreated control. These concentrations were equivalent to 0.46, 1.0, 2.14, 4.56, 9.97 mg/L of WRS-2390TX (corrected for 71.2% test item purity).

Samples were taken for chemical analysis at initiation and completion of the exposure phase. The corresponding mean measured concentrations were 0.36, 0.79, 1.72, 3.77, 8.88 mg/L, which was equivalent to 78, 79, 80, 83 and 89% of the nominal values, corrected for purity. Results are based on mean measured concentration but the concentrations at which effects were observed were closest to nominal and within 20% of the expected concentration.

For the definitive test immobilisation observed for each treatment group at 48 h was as follows:

Measured Concentration (mg/L)	Immobilisation (%)
Control	0
0.36	10
0.79	0
1.72	0
3.77	20
8.88	100

The 48 h EC_{50} for WRS-2390TX to *Daphnia* was estimated as 4.2 mg/L. The 95% confidence limits could not be calculated.

The 48 h No Observed Effect Concentration (NOEC) for WRS-2390TX to *Daphnia* was 1.72 mg/L.

Water quality parameters (pH, temperature, conductivity and dissolved oxygen concentration) remained within acceptable limits over the definitive test: pH 7.6-7.7; temperature 19.6-21.0°C; conductivity 652-687µS/cm and dissolved oxygen 80.7-93.2% ASV (air saturation value).

2 Introduction

The median effective concentration (EC_{50}) for immobilisation of *Daphnia magna* exposed to WRS-2390TX was determined over a 48 h period. The study was conducted under static conditions in accordance with OECD (1984) Guideline 202 Part I and EC (2001) Guideline C2.

The study was initiated on 02 December 2002 and was conducted at Inveresk, Tranent, EH33 2NE, Scotland. Experiments commenced on 25 February 2004 and were completed on 29 March 2004. For study completion date see date of Study Director's Authentication.

All data generated and recorded during this study, including a copy of the final report, will be stored in the Scientific Archives of Inveresk for 5 years after issue of the final report. After the 5 year period the Sponsor will be consulted regarding the disposal, transfer or continued storage of raw data.

3 Experimental Procedure

3.1 Test Item

Identification:	WRS-2390TX
Appearance:	Brown viscous liquid
Notebook No.:	02-181-126
CAS No.:	68227-46-3
Purity:	71.2%* (in water)
Receipt Date:	02 October 2003
Expiry Date:	15 November 2004
Storage:	Under ambient temperature, in the dark.

* A specific purity was supplied for this material by the Sponsor for this test item with a reference of Notebook No. 02-181-126.

The test item is a Propanoic acid, 2-hydroxy- compound with 3-[2-(dimethylamino)ethyl]1-(2ethylhexyl)(4-methyl-1,3-Phenylene)bis[carbamate] (1:1).

3.2 Test Species

Daphnia magna (water flea) were used for this study. They were bred within the laboratory by acyclical parthenogenesis and the neonates used were <24 h old at study initiation. *Daphnia* holding cultures were maintained in a synthetic medium (Elendt M4).

3.3 Food and Culture Medium

Daphnia cultures were fed on a diet of *Chlorella vulgaris*. *Daphnia* were not fed during the test. *Daphnia* cultures were maintained in a synthetic medium (Elendt M4) and all testing was conducted using this medium (Appendix 2).

3.4 Test Vessels

The test was conducted using glass crystallising dishes (*ca* 200 ml), covered with perspex lids to prevent dust contamination and evaporation loss.

3.5 Range Finding Test

A range finding test was conducted over 48 h, at the following nominal concentrations of WRS-2390TX without adjustment for purity:

0.1, 1.0, 10, and 100 mg/L, with an untreated control.

Test solutions were prepared by the serial dilution of a 100 mg/L WRS-2390TX stock solution with Elendt M4 *Daphnia* medium. The 100 mg/L solution was prepared by adding a weighed amount of WRS-2390TX (100.7 mg) to Elendt M4 and bringing to volume in a 1000 ml volumetric flask. This was then placed in an ultrasonic bath for *ca* 10 min to aid dissolution.

Duplicate vessels were prepared for each treatment group, each containing 100 ml of test solution. Five *Daphnia* neonates <24 h old were then added to each vessel.

3.6 *Definitive Test*

Based on the results of the range finding test and discussions with the Sponsor, a static exposure definitive test was conducted over 48 h with an untreated control at the following concentrations of WRS-2390TX:

0.64, 1.4, 3.0, 6.4, 14 mg/L

These concentrations were equivalent to the following concentrations when corrected for the purity of the test item:

0.46, 1.0, 2.14, 4.56, 9.97 mg/L

Test solutions were prepared by parallel dilution of a 100 mg/L stock solution. The stock solution was prepared by adding a weighed amount of WRS-2390TX (25.1 mg) to Elendt M4 and bringing to volume in a 250 ml volumetric flask. This was then placed in an ultrasonic bath for *ca* 10 min to aid dissolution.

Four vessels were prepared for each treatment group and control, each containing 100 ml of media. Five *Daphnia* were added to each vessel.

3.7 *Observations*

Test vessels were observed for any immobile *Daphnia* at 24 and 48 h after test initiation. *Daphnia* were recorded as immobile if no movement was observed within 15 s following gentle agitation of the test vessel.

3.8 *Determination of WRS-2390TX in Test Solutions*

Duplicate *ca* 20 ml samples were taken from freshly prepared test item and control media for chemical analysis at test initiation (0 h) and from pooled samples from all replicates at 48 h in both test phases of the study. In the range finding test, only samples from the highest and lowest concentration plus controls were taken. In the definitive test samples from all test and control solutions were taken.

The test solutions were analysed according to the procedures established and validated under Inveresk Study No. 342753 (Appendix 1).

3.9 *Statistical Analysis*

Statistical analysis of the observed immobilisation data at each time point compared to the nominal concentration was conducted by probit (Finney 1971, 1978) transformation applied to the mortality data.

A Pearson Chi-square test on the sum of squares for each data point indicated low heterogeneity in the data. The probit transformed data were subjected to a regression procedure against logarithmically transformed concentrations where appropriate. The Davidon-Fletcher-Powell maximum likelihood algorithm was used to obtain parameter estimates but there was poor fit to the model as the number of intermediate values was limited. The EC_{50} value was estimated from the fitted model but was not reliable and no confidence limits were presented.

3.10 Water Quality Parameters

Water quality parameters were measured in one vessel for each treatment group at 24 h intervals during the definitive test, using a Sentron Argus X pH meter, a Jenway 470 conductivity meter and a YSI 550A dissolved oxygen meter.

3.11 Environmental Control

Test vessels were maintained within a temperature-controlled laboratory with the aim of achieving a temperature in the range 18-22 °C. The temperature did not deviate by more than 2 °C throughout the test. A light cycle of 16 h light and 8 h dark was in operation throughout the test and provided by artificial daylight fluorescent tubes. Vessels were not aerated during the test.

4 Results and Discussion

4.1 Range Finding Test

Analysis of samples at 0 h in the range finding indicated that samples had been accurately prepared. At 48 h samples showed a loss of measured test item at both the high and low concentrations analysed (see Table 1). It is unclear why concentrations were not maintained over 48 h in the range finding test. It was noted that the 100 mg/L solution was opaque white but all other test solutions were clear and colourless.

Immobilisation observed during the range finding test is presented in Table 2. One replicate vessel of the control treatment *Daphnia* neonates were immobilised. The reason for this is not known. However this did not impede the selection of a suitable test range for the definitive test. After 24 h 100% of neonates at 100 mg/L were immobile and after 48 h 100% of neonates at 10 mg/L were immobile. No immobile neonates were observed at 1 mg/L.

In the 0.1 mg/L group, 3/10 neonates were immobile at 48 h. This was not regarded as a test item effect as it did not follow a normal concentration response.

4.2 Definitive Test

Table 3 gives a summary of the analytical results. These indicate that test solutions were either equal to or greater than 80% of nominal (corrected for purity) for the highest three concentrations at preparation. The lower concentrations were 78 and 79% of nominal. However results from 48 h show that the concentrations were stable over the test period. Mean measured concentrations for the exposure period were 0.36, 0.79, 1.72, 3.77 and 8.88 mg/L, which were between 78 and 89% of nominal. As the mean measured test item concentrations deviated by >20% from nominal, test results will be based on mean measured concentration.

All prepared test solutions were clear and colourless at test initiation. No change was noted at 48 h.

Table 4 shows observed immobilisation for the definitive test. Effects were observed after 24 h at the highest concentration only, 8.88 mg/L measured, where 20% (4 *Daphnia*) were immobilised. After 48 h, 100% immobilisation (20 *Daphnia*) was observed at the 8.88 mg/L treatment with 20% immobilisation (4 *Daphnia*) at the 3.77 mg/L treatment.

At 48 h 10% immobilisation (2 *Daphnia*) was observed at the lowest concentration tested, 0.36 mg/L. This is not regarded as an effect of the test item, as this is within the permitted effect levels for controls and does not follow a standard concentration response.

From these observations the following results were determined:

The 24 h EC_{50} was >8.88 mg/L, the highest concentration tested in the definitive test.

The 48 h EC_{50} was 4.2 mg/L. As there were limited intermediate levels of effect, the probit model was not a good fit to the observed data, so the estimated value should be regarded with caution and 95% confidence limits were not calculated.

The 48 h No Observed Effect Concentration (NOEC) was 1.72 mg/L (measured), under the conditions of the test.

4.3 Environmental Conditions

Water quality parameters (pH, temperature, conductivity and dissolved oxygen concentration) remained within acceptable limits over the definitive test: pH 7.6-7.7, temperature 19.6-21.0°C; conductivity 652-687 µS/cm and dissolved oxygen 80.7-93.2% ASV (air saturation value).

Water quality parameters recorded during the definitive test are presented in Table 5.

Water hardness was determined in a sample of the medium used to prepare the test solutions at initiation of the definitive test as 270 mg $CaCO_3$/L. .

5 Conclusion

The 24 h EC_{50} for WRS-2390TX to *Daphnia magna* was >8.88 mg/L (measured), the highest concentration tested.

The 48 h EC_{50} for WRS-2390TX to *Daphnia magna* was estimated as 4.2 mg/L (measured), though 95% confidence limits could not be calculated as the probit model did not have a good fit to the observed data.

The 48 h NOEC for WRS-2390TX to *Daphnia magna* was 1.72 mg/L.

6 References

EC (2001) 92/69/EEC, Method for the Determination of Ecotocicity, C.2, Acute Toxicity for *Daphnia*.

Finney, D. J. (1971) Probit Analysis. 3rd Ed. Carles Griffin & Company, London.

Finney, D. J. (1978) Statistical Method in Biological Assay. 3rd Ed. Charles Griffin & Company, London.

OECD Paris (1984), OECD Guideline for Testing of Chemicals, Test Guideline 202, '*Daphnia* sp. Acute Immobilisation Test and Reproduction Test', Part 1.

7 Tables

Table 1 Measured Concentrations in the Range Finding Test

Nominal concentration (mg/L)	Equivalent concentration corrected for purity (mg/L)	Mean Concentration at 0h (mg/L) / nominal %	Mean Concentration at 48 h (mg/L) / nominal %	Mean measured concentration (mg/L) / nominal %
Control	NA	ND	ND	ND
0.1	0.071	0.06 / 85	0.05 / 64	0.06 / 77*
100	71.2	65.6 / 92.2	46.1 / 65	55.9 / 79

LOQ = 0.04 mg/L

NA Not applicable
ND Not detected

* based on 0.055 mg/L

(purity = 71.2%)

Table 2 ***Immobilisation Observed in the Range Finding Test***

Time (h)	Vessel Replicate	Nominal Concentration of WRS-2390TX (mg/L)				
		Control	0.1	1	10	100
24	A	5*	0	0	0	5
	B	0	0	0	0	5
	% Immobile *Daphnia*	50	0	0	0	100
48	A	5*	3	0	5	5
	B	0	0	0	5	5
	% Immobile *Daphnia*	50	30	0	100	100

*Total loss of control Daphnia for unknown reason – did not affect definitive concentration range selection.

Table 3 ***Measured Concentrations in the Definitive Test***

Nominal Concentration* (mg/L)	Time (h)	Mean Measured Concentration (mg/L)	Mean Measured Concentration (mg/L)	Overall mean measured concentration (mg/L)	Percentage of nominal (%)
Control	0	ND ND	ND	NA	NA
	48	ND ND	ND		
0.46	0	0.35 0.35	0.35	0.36	78
	48	0.38 0.35	0.37		
1.0	0	0.80 0.75	0.78	0.79	79
	48	0.80 0.78	0.79		
2.14	0	1.75 1.70	1.73	1.72	80
	48	1.70 1.70	1.70		
4.56	0	3.70 3.65	3.68	3.77	83
	48	3.90 3.80	3.85		
9.97	0	8.80 8.90	8.85	8.88	89
	48	8.80 9.00	8.90		

* Corrected for test item purity

LOQ = 0.04 mg/L

ND = Not detected
NA = Not applicable

Table 4 ***Immobilisation Observed in the Definitive Test***

Time (h)	Vessel Replicate	Mean Measured Concentration of WRS-2390TX (mg/L)					
		Control	0.36	0.79	1.72	3.77	8.88
24	I	0	0	0	0	0	0
	II	0	0	0	0	0	0
	III	0	0	0	0	0	2
	IV	0	0	0	0	0	2
	% Immobile *Daphnia*	0	0	0	0	0	20
48	I	0	0	0	0	1	5
	II	0	1	0	0	0	5
	III	0	1	0	0	2	5
	IV	0	0	0	0	1	5
	% Immobile *Daphnia*	0	10^	0	0	20	100

^ Not regarded as test item related

Table 5 ***Water Quality Parameters Recorded in the Definitive Test***

Time (h)	Parameter	Mean Measured Concentration of WRS-2390TX (mg/L)					
		Control	0.36	0.79	1.72	3.77	8.88
0	pH	7.7	7.7	7.7	7.7	7.7	7.6
	Temperature (°C)	21.0	20.8	20.8	20.7	20.7	20.6
	Conductivity (µS/cm)	655	653	655	655	655	656
	Dissolved Oxygen (%)*	88.4	86.1	93.2	90.2	88.5	88.1
24	pH	7.7	7.7	7.7	7.7	7.7	7.7
	Temperature (°C)	19.6	19.7	19.8	19.8	19.8	19.9
	Conductivity (µS/cm)	658	652	652	657	660	658
	Dissolved Oxygen (%)*	80.7	85.2	87.1	84.3	87.1	87.3
48	pH	7.7	7.7	7.7	7.7	7.7	7.7
	Temperature (°C)	20.7	20.8	20.9	20.9	20.8	20.8
	Conductivity (µS/cm)	687	685	686	685	686	682
	Dissolved Oxygen (%)*	82.1	82.5	89.4	88.7	87.5	89.4

* = % air saturation value

8 Appendices

Appendix 1 Analytical Method

8 Appendix

Appendix 1 Analytical Method No. 4275

Assay: HPLC (Range 1.42 mg.l^{-1} to 0.036 mg.l^{-1})	Analytical Method No.: 4275
Test Item: WRS-2390	Date of Approval: 05 December 2003
	Approved: A Hogg

I. Summary

Solutions of WRS-2390 are assayed by high performance liquid chromatography. Detection is by uv and external standardisation is used. Other instrumentation may be used providing adequate accuracy and precision can be achieved.

II. Reagents

a) WRS-2390 (purity 71.2 %).
b) Phosphoric acid.
c) Milli Q water or equivalent grade.
d) Acetonitrile, HPLC grade.
e) Milli RO water or equivalent.

III. Apparatus

a) LC Module 1, supplied by Waters or equivalent.
b) Balance, 5 figure.
c) Laboratory glassware.
d) Volumetric glassware.
e) Sonic bath.

IV. Chromatographic Conditions/Set Up

HPLC: Waters LC Module 1.
Column: YMC ODS-AM, 150 mm x 4.6 mm, 3 µm.
Column temperature: 40°C.
Mobile phase A: 0.1 % phosphoric acid in Milli Q water.
Mobile phase B: acetonitrile.
Detection: uv at 225 nm.
Injection volume: 150 µl.
Run time: 20 min.

Appendix 1 (continued) Analytical Method

Appendix 1 (continued)

Flow rate: 1 $ml.min^{-1}$.
Needle wash: Milli Q water/acetonitrile (9:1, v/v).
Data collection: LabSystems Vax Multichrom 2 version 2.30b or other suitable data collection system.
Gradient timetable:

Time (min)	% Mobile Phase A	% Mobile Phase B	Curve
0	90	10	-
1	90	10	6
10	5	95	6
12	5	95	6
12.10	90	10	6
20	90	10	6

V. Preparation of Standard Curve

Accurately weigh ca 50 mg of WRS-2390 into a 100 ml volumetric flask then dissolve and make to volume using acetonitrile to give a *ca* 500 $mg.l^{-1}$ solution. Sonicate to aid dissolution. Dilute the *ca* 500 $mg.l^{-1}$ solution by adding 2.5 ml to a 50 ml volumetric flask and make to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) to give a *ca* 25 $mg.l^{-1}$ solution. Dilute this solution as detailed in the following table:

Standard Identity	Concentration of Stock Solution used ($mg.l^{-1}$)	Volume Diluted (ml)	Final Volume (ml)	*Nominal Concentration ($mg.l^{-1}$)
B1	25	4	50	1.42
B2	25	3	50	1.07
B3	25	2	50	0.71
B4	25	1	50	0.36
B5	25	1	100	0.18
B6	2	5	100	0.071
B7	2	2.5	100	0.036

* corrected for test item purity (71.2 %)

Make each standard to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v).

Note: Alternative dilutions may be performed providing the sample final concentration is achieved.

Analyse each standard solution, in duplicate, according to the chromatographic conditions detailed in Section IV.

Appendix 1 (continued) Analytical Method

Appendix 1 (continued)

VI. Preparation of Quality Control Sample

Accurately weigh *ca* 50 mg of WRS-2390 into a 100 ml volumetric flask then dissolve and make to volume using acetonitrile to give a *ca* 500 $mg.l^{-1}$ solution. Sonicate to aid dissolution. Dilute the *ca* 500 $mg.l^{-1}$ solution by adding 2.5 ml to a 50 ml volumetric flask and make to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) to give a *ca* 25 $mg.l^{-1}$ solution. Dilute this solution by adding 2 ml to a 50 ml volumetric flask and make to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) to give a *ca* 1 $mg.l^{-1}$ (0.71 $mg.l^{-1}$ purity corrected) solution.

Note: Alternative dilutions may be performed providing the sample final concentration is achieved.

Analyse the *ca* 1 $mg.l^{-1}$ (0.71 $mg.l^{-1}$ purity corrected) solution, in duplicate, according to the chromatographic conditions detailed in Section IV.

VII. Preparation of Aqueous Samples for Analysis

Aqueous solutions of WRS-2390 in Milli RO water or algae or daphnia media may be directly injected onto the analytical column and analysed according to the chromatographic conditions detailed in Section IV, providing the sample concentration is within the range of the standard curve detailed in Section V.

If required, aqueous solutions of WRS-2390 in Milli RO water or algae or daphnia media may be diluted to within the range of the standard curve detailed in Section V using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v).

Note: The method validation indicated that direct injections of WRS-2390 in aqueous media resulted in *ca* 82 % recoveries from the nominal applied concentration. If possible, aqueous samples should be diluted using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) prior to analysis. Otherwise the accuracy of the results should be quoted as 100 % ± 20 % of the found concentration.

The diluted samples should be analysed, in duplicate, according to the chromatographic conditions detailed in Section IV.

Appendix 2 Elendt M4 Medium

Elendt M4 medium was prepared as follows:

Separate stock solutions (I) of individual trace elements are prepared in deionised water. From these, a second single stock solution (II) is prepared containing all the trace elements.

Component	Stock Solution I (mg/L)	Volume of Stock Solution I used to prepare Stock Solution II
H_3BO_3	57190	1.0
$MnCl_2.4H_2O$	7210	1.0
LiCl	6120	1.0
RbCl	1420	1.0
$SrCl_2.6H_2O$	3040	1.0
NaBr	320	1.0
$Na_2MoO_4.2H_2O$	1260	1.0
$CuCl_2.2H_2O$	335	1.0
$ZnCl_2$	260	1.0
$CoCl_2.6H_2O$	200	1.0
KI	65	1.0
Na_2SeO_3	43.8	1.0
NH_4VO_3	11.5	1.0
$Na_2EDTA.2H_2O$	5000	20.0*
$FeSO_4.7H_2O$	1991	

* = The Na_2EDTA and $FeSO_4$ solutions are prepared separately, combined and autoclaved to produce Fe-EDTA. The volumes indicated of the combined solution are added to Stock Solution II.

A stock solution of vitamins is prepared in deionised water from the following components:

Vitamin	Concentration (mg/L)
Thiamine hydrochloride	750
Cyanocobalamine	10
Biotin	7.5

The media is prepared from the trace elements in Stock Solution II, the vitamins stock solution, and stock solutions in deionised water of individual macro-nutrients as indicated below:

Appendix 2
(continued)

Component	Stock Solution I (g/L)	Volume of Stock Solution I in Final Medium ($ml.l^{-1}$)
Trace elements Stock Solution II	-	50
Vitamins Stock Solution	-	0.1
$CaCl_2.2H_2O$	293.8	1.0
$MgSO_4.7H_2O$	246.6	0.5
KCl	58.0	0.1
$NaHCO_3$	64.8	1.0
$Na_2SiO_3.9H_2O$	50.0	0.2
$NaNO_3$	2.74	0.1
KH_2PO_4	1.43	0.1
K_2HPO_4	1.84	0.1

To avoid precipitation of salts when preparing the final medium, aliquots of the stock solutions are added to *ca* half the final volume of deionised water, then made up to the final volume.

The medium was continuously aerated until use, after which the pH and hardness were determined as 7.8 and 280 mg $CaCO_3$/L (range finding test) and 7.7 and 270 mg $CaCO_3$/L (definitive test) respectively.

Appendix 3 GLP Certificate

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
Inveresk Research International Ltd. **Elphinstone Research Centre** **Tranent** **East Lothian** **EH33 2NE**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Toxicity** **Environmental Fate** **Mutagenicity** **Phys/chem Tests** **Toxicology**
Including	
Veterinary Clinical Trials Unit **Talkin** **Brampton** **Cumbria** **CA8 1LE**	**Safety studies on Veterinary products**

DATE OF INSPECTION

13 April 2004

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority

CONFIDENTIAL

Inveresk Report Number 23966

WRS-2390TX
Alga, Growth Inhibition Test (96 h, EC_{50})

Authors:

T L Hargreaves
C R Kelly

Sponsor:

PPG Industries Inc
4325 Rosanna Drive
P O Box 9
Allison Park
Pennsylvania 15101
USA

Performing Laboratory:

Inveresk
Tranent
EH33 2NE
Scotland

Final Page of Report: 40

284536

Contents

Authentication

'I, the undersigned, hereby declare that this work was performed in accordance with the OECD Principles of Good Laboratory Practice as set forth by the United Kingdom Department of Health and as accepted by Regulatory Authorities throughout the European Community , United States of America (FDA and EPA) and Japan (MHLW, MAFF and METI). The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained.'

T L Hargreaves BSc
Study Director

Date

Quality Assurance Statement

The execution of this type of short-term study is not individually inspected. The processes involved are inspected by the Inveresk Quality Assurance Unit at intervals according to a predetermined schedule.

Date of QA Inspection	Phase	Date of Report to Management
24 March 2004	Addition of Test Item / Addition of Test Organisms / Preparation of Vessels / Water Quality Parameters	25 March 2004
25 February 2004	Protocol Review	26 February 2004

The protocol review was also reported to the Study Director.

The report has been audited by the Quality Assurance Personnel according to the appropriate Standard Operating Procedure(s). The report is considered to describe accurately the methods and procedures used in the study. The reported results accurately reflect the original data generated during the study.

D Allan BSc
Quality Assurance

Date

Personnel Involved

Study Director:	T L Hargreaves BSc (28 July 2004 – Study Completion)
	C R Kelly BSc PhD (17 February 2004 – 27 July 2004)
Head, Environmental Sciences:	B E Hall BSc PhD
Report Compilation:	T L Hargreaves BSc C R Kelly BSc PhD
Scientific Assistance:	C M Murphy BSc
Chemical Analysis	A Hogg BSc W Brown BSc
Statistical Analysis	G J Hunter BSc MSc S E Laing BSc PgDip
Quality Assurance:	D Allan BSc

1 Summary

The median effect concentration (EC_{50}) for growth of *Pseudokirchneriella subcapitata*, a unicellular freshwater green alga, exposed to WRS-2390TX was determined over a 96 h period, based on the procedures described in EPA (1996) Ecological Effects Test Guidelines OPPTS 850.5400 and OECD Draft Revised Guideline 201 (2002).

The definitive test was conducted over two phases. The first phase was the exposure phase, conducted with three replicate vessels at each test concentration and six replicate vessels for the untreated control. Concentrations for the exposure phase of the definitive test were selected based on the results of range finding tests. The test concentrations selected were 0.04, 0.09, 0.20, 0.43 and 0.94 mg/L total test item. These concentrations were equivalent to 0.03, 0.06, 0.14, 0.31 and 0.67 mg/L (corrected for 71.2% test item purity).

Samples were taken for chemical analysis at initiation and completion of the exposure phase. No test item was detected in samples from untreated control vessels during the test. The mean measured concentrations from vessels containing test item at the initiation of the exposure phase (0 h) were 0.03, 0.06, 0.14, 0.32 and 0.67 mg/L. These were all 100 to 103% of nominal values, when corrected for purity. After 96 h exposure, the measured test concentrations were 0.01, 0.02, 0.09, 0.22 and 0.56 mg/L, which corresponded to 33, 33, 64, 71 and 84% of nominal values, when corrected for purity. As the measured concentrations of test item were clearly reduced compared to nominal all results are based on the geometric mean of the initial and 96 h analytical concentrations. Geometric means were calculated as 0.02, 0.03, 0.11, 0.27 and 0.61 mg/L, respectively.

Samples from satellite vessels prepared at nominal concentrations of 0.06 and 0.67 mg/L, prepared without algae, gave measured concentrations of 76 and 83%, respectively, at completion of the exposure phase. It was noted that the lowest percent recoveries from analytical samples, at exposure termination, occurred at the lowest concentrations where algal cell division and growth was highest. These observations suggest that the test item may have been absorbed/adsorbed to the biomass of algal cells.

Statistical analysis of daily cell counts was conducted. The 96 h EC_{50} for WRS-2390TX, based on Area Under the Curve (AUC), was determined to be 0.07 mg/L (measured). The 96 h EC_{50} for WRS-2390TX, based on Growth Rate, was determined to be 0.16 mg/L (measured).

The No Observed Effect Concentration at 96h based on AUC was <0.02 mg/L, the lowest geometric mean measured concentration tested, where as the NOEC was 0.11 mg/L (measured) based on the growth rate.

The second phase of the definitive test was the re-inoculation phase. At completion of the exposure phase samples were taken from the control replicates and replicate flasks of any concentration in which cell numbers were <50% of the cell density of the controls (0.27 and 0.61 mg/L, corrected for purity). These were inoculated into fresh

medium at the rate of 1:19 (exposure medium/fresh medium) and incubated for 9 days under similar conditions to the exposure phase. Observations and cell counts were made at growth rates compared statistically. This indicated that the effect of the test item to algal cells was phytostatic and not phytotoxic, as no significant differences were found in growth rate after 9 days. However the cells in the two test item treatments were both noted to have formed clumps visible to the naked eye, compared to the controls where growth was normal.

2 Introduction

This study was designed to determine the median effect concentration (EC_{50}) for growth of a unicellular green alga species exposed to WRS-2390 TX. Exponentially-growing cultures of the green alga *Pseudokircherniella subcapitata* (formally known as *Selenastrum capricornutum)* were exposed to various concentrations of the test item over several generations under defined conditions. The inhibition of growth in relation to a control culture was determined over a fixed period of 96 h.

The study was prepared in order to meet the requirements of EPA Ecological Effects Test Guidelines OPPTS 850.5400 Algal Toxicity, Tiers I and II, Public Draft (April 1996). It also followed the general principles of OECD Draft Revised Guideline 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2002).

The study was initiated on 17 February 2004 and was conducted at Inveresk, Tranent, EH33 2NE, Scotland. Experiments commenced on 23 February 2004 and were completed on 15 April 2004. For study completion date see date of Study Director's Authentication.

All data generated and recorded during this study, including a copy of the final report, will be stored in the Scientific Archives of Inveresk for 5 years after issue of the final report. After the 5 year period the Sponsor will be consulted regarding the disposal, transfer or continued storage of raw data.

3 Experimental Procedure

3.1 Test Item

Identification:	WRS-2390TX
Appearance:	Brown viscous liquid
Notebook No.:	02-181-126
CAS No.:	68227-46-3
Purity:	71.2%* (in water)
Receipt Date:	02 October 2003
Expiry Date:	15 November 2004
Storage:	Under ambient temperature, in the dark.

* A specific purity was supplied for this material by the Sponsor for this test item with a reference of Notebook No. 02-181-126.

The test item is a Propanoic acid, 2-hydroxy- compound with 3-[2-(dimethylamino)ethyl]1-(2ethylhexyl)(4-methyl-1,3-Phenylene)bis[carbamate] (1:1).

3.2 Test Species

Pseudokirchneriella subcapitata were used for this study as it is recommended in the guidelines. The test species was supplied from Culture Centre of Algae and Protozoa (CCAP), Cumbria, England.

The algae pre-culture used for inoculation during the definitive test was healthy and uninfected. The pre-culture was held for 1 day, rather than 3-7 days as stated in the protocol (protocol deviation), in conditions similar to those used in the test. This pre-culture was initiated from a culture (incubated for 3 days) which was initiated directly from a refrigerated slope, supplied by CCAP.

3.3 Test Vessels

Glass Erlenmeyer flasks (250 mL) were used as the test vessels. Each test vessel was labelled with the project number, vessel/replicate number, test concentration and study start date.

3.4 Environmental Control

The cultures and test vessels were maintained within an illuminated cooled orbital shaking incubator at a temperature range of 22-26 °C. The temperature was monitored continuously in a single control vessel for the duration of the definitive test.

The algae were provided with continuous uniform illumination obtained from universal white-type fluorescent lamps.

Light intensity on the shaker table was checked at 3 different places prior to the start of the test using a Lutron LX-101 Lux meter. This ranged from 4000 to 4300 lux in line with protocol requirements.

3.5 *Test Medium Preparation*

The dilution and control medium for both the range finding and definitive tests was ISO freshwater algal medium. The stock solutions were made up to volume in 0.2 µm filtered deionised water (Appendix 1). The pH of the medium used to prepare the test solutions was measured at the beginning of both the range finding and definitive tests.

3.6 *Initial Cell Concentration*

The initial cell concentration in the test cultures was *ca* 1 x 10^4 cells/mL.

3.7 *Failed Range Finding Test*

An initial range finding test was conducted at 1.0, 10, 100 and 1000 mg/L with an untreated control. No growth was found at any test concentration and this test was repeated at the lower concentration range to try to establish a definitive test concentration range. These data are retained in the study file and are not reported.

3.8 *Range Finding Test*

A second range finding test was conducted under static conditions over a 96 h period at nominal WRS-2390TX formulation concentrations of 0.001, 0.01, 0.1 and 1.0 mg/L. An untreated control was also tested.

A 1.0 mg/L test item solution was prepared by adding a weighed amount of test item (*ca* 2 mg) to a 2 litre capacity volumetric flask and bringing to volume with ISO freshwater algal medium. This solution was sonicated for *ca* 10 min to aid dissolution. Aliquots of the 1.0 mg/L solution (0.25, 2.5 and 25 mL) were placed into 250 mL capacity volumetric flasks and made up to volume with ISO freshwater algal medium to prepare the 0.001, 0.01 and 0.1 mg/L test media, respectively.

Duplicate flasks were prepared for each treatment group and control, each containing 100 mL of test solution or untreated medium, as appropriate. The flasks were then inoculated with *ca* 1 x 10^4 cells/mL *Pseudokirchneriella subcapitata*.

Algal cell concentrations were determined in each test flask after 96 hours.

3.9 *Definitive Test*

Based on the results of the second range finding test, a definitive test was conducted over 96 h (exposure phase) and included a re-inoculation phase of 9 days. The definitive test was conducted with nominal WRS-2390TX concentrations of 0.04, 0.09, 0.20, 0.43 and 0.94 mg/L total test item; these concentrations were equivalent to 0.03, 0.06, 0.14, 0.31 and 0.67 mg/L (corrected for 71.2% test item purity). An untreated control was also tested.

A 100 mg/L test item solution was prepared by adding a weighed amount of test item (*ca* 10 mg) to a 100 ml capacity volumetric flask and bringing to volume with ISO freshwater algal medium. This solution was sonicated for *ca* 10 min to aid dissolution. Aliquots of the 100 mg/L test item solution (0.2, 0.45, 1.0, 2.15 and 4.7 mL) were brought to volume with ISO freshwater algal medium in 500 ml volumetric flasks to prepare the 0.04, 0.09, 0.20, 0.43 and 0.94 mg/L treatment groups, respectively.

Flasks were prepared in triplicate for each treatment group, with six flasks prepared for the control. Each flask contained 100 mL of media and was inoculated with *Pseudokirchneriella subcapitata*. Algal cell concentrations were determined in each test flask at 24, 48, 72 and 96 h during the definitive test.

From visual observations and cell concentration determination it was determined that the 0.94 and 0.43 mg/L treatment groups had >50% inhibition of growth after 96 h exposure to WRS-2390TX. These were inoculated into fresh medium at the rate of 1:19 (exposure medium/fresh medium) and incubated for 9 days with the re-inoculated controls under similar conditions to the exposure phase

Algal cell concentrations were determined in each test flask at 24, 48, 72 and 96 hours during the exposure phase of the definitive test and on Day 9 of the re-inoculation phase. Samples (2 mL) were removed from each flask and combined with 20 µL Lugol's Iodine Solution. The samples were then shaken by hand, to ensure thorough mixing with the iodine, and stored at *ca* 4°C until determination of cell concentration. Cell concentrations were determined within 7 days after sampling using a Compound Light Microscope and Improved Neubauer Counting Chambers.

An extra flask was prepared at the 0.94 and 0.09 mg/L treatment groups. These flasks were not inoculated with algal cells. Chemical analysis of the medium in these flasks, compared with the analysis of samples from inoculated flasks, indicate if adsorption/absorption to the algal cells occurred over the exposure period.

The pH of the prepared test item stock solutions were determined at the beginning of the definitive test. In addition, the pH of every test flask was determined after the 96 h exposure phase.

3.10 Determination of WRS-2390TX Concentration in Test Samples

The concentration of test item in test samples was analysed at Inveresk using a method validated under Inveresk Project No. 342753 (Appendix 2).

At the start of the initial range finding test and after 96 h exposure, samples were taken at the highest test concentration, the lowest concentration and control vessels for analysis. No analysis samples were collected or analysed for the second range finding test, conducted at lower concentrations.

In the definitive test exposure phase, samples for analysis were taken from all concentrations and the controls at the start of the study and after 96 h. Upon receipt in

the analytical laboratories, the samples taken at 96 h were centrifuged at 1000 rpm for 5 min to remove algal cells. The remaining supernatant from each sample was analysed.
Analysis was normally performed on the same day but when this was not possible the samples were stored under appropriate conditions until analysis could take place.

3.11 Statistical Analysis

The daily cell counts for the exposure phase were tabulated with the geometric mean measured concentrations at each test concentration for comparison and analysis. The geometric mean measured concentration was used as there was substantial reduction of test concentration after the 96 h exposure. The geometric mean of 0h and 96 h results is taken as the reasonable worst case scenario for estimating the concentration at which effects were brought about over the exposure period.

Analysis of data from the re-inoculation phase was undertaken to observe any effects on growth rate. Growth rate was taken as the best measure of effects after re-inoculation as this remained unaffected by initial cell density.

4 Results and Discussion

4.1 Range Finding Test

The results of the range finding test indicate that growth was inhibited by 16.6, 5.2, 16.3 and 99.3% at 0.001, 0.01, 0.1 and 1.0 mg/L WRS-2390TX, respectively, as indicated by cell numbers when compared with control cultures (Table 1).

4.2 Definitive Test

Based on the results of the range finding test, a definitive test was conducted over 96 h (exposure phase) plus a re-inoculation phase of 9 days.

The first phase (exposure phase) was conducted with three replicate vessels at each test concentration and six replicate vessels for the untreated control. The test concentrations selected were: 0.04, 0.09, 0.20, 0.43 and 0.94 mg/L total test item, which were equivalent to nominal concentrations of 0.03, 0.06, 0.14, 0.31 and 0.67 mg/L (corrected for 71.2% test item purity).

4.2.1 Determination of WRS-2390TX Concentration in Test Samples

Samples were taken for chemical analysis at initiation and completion of the exposure phase (Table 2). The mean measured concentrations from vessels containing test item concentrations at the initiation of the exposure phase (0 h) were 0.03, 0.06, 0.14, 0.32 and 0.67 mg/L. These values were 100 to 103% of the nominal values when corrected for purity. After 96 h exposure, the measured test concentrations were 0.01[1], 0.02[1], 0.09, 0.22 and 0.56 mg/L, which corresponded to 33, 33, 64, 71 and 84% of nominal values when corrected for purity.

As the measured concentrations of test item were clearly reduced compared to nominal all results are based on the geometric mean of the initial and 96 h analytical concentrations. Geometric means were calculated as 0.02, 0.03, 0.11, 0.27 and 0.61 mg/L, respectively.

Samples from satellite vessels prepared at nominal concentrations of 0.06 and 0.67 mg/L, prepared without algae, gave measured concentrations of 76 and 83%, respectively, at completion of the exposure phase. It was noted that the lowest percent recoveries from analytical samples, at exposure termination, occurred at the lowest concentrations where algal cell division and growth was highest. These observations suggest that the test item may have been absorbed/adsorbed to the biomass of algal cells.

Details of the analytical method are given in Appendix 2.

[1] These concentrations (0.01 and 0.02 mg/L) were lower than the lowest analytical standard prepared (0.03 mg/L) and have been calculated by linear extrapolation.

4.2.2 *Exposure Phase Growth*

Daily cell counts, expressed as cells per mL, are given in Table 3. The cell density data was used to calculate Area Under the Curve (AUC) biomass estimates (Table 4) and growth rate (Table 5) estimates. These were used in the statistical analysis (Appendix 3) to give end points for the exposure phase.

Figure 1 shows mean AUC values for each day for all test concentrations and the control. This clearly shows there was no growth at the highest concentration (0.61 mg/L) and that the 0.27 mg/L cultures were growing for the first 48 h followed by a reduction in biomass. Growth in the control cultures was strong over the 96 h period.

Figures 2 and 3 examine the inhibition of growth at each day for AUC (Figure 2) and growth rate (Figure 3) across the test range compared to the control. The key concentration for both graphs appeared to be the 0.11 mg/L test group. For this concentration the highest rate of inhibition for both measures occurred within the first 24 to 48 h. Data at 96 h, for this concentration, was the least inhibited time point. This suggests that these cultures recovered from initial exposure to the test item. It was likely this was linked to the apparent adsorption/absorption indicated in the analysis. Algal cells may have reduced the concentration of available test item, allowing new cells to grow and the culture to recover. Results in Table 2 show measured concentrations at the initiation of the test at 0.14 mg/L whereas this was reduced to 0.09 mg/L after 96 h (a *ca* 36 % reduction in concentration) which was lower than the NOEC for growth rate determined in this test (see Section 4.2.3).

The pH recorded during the definitive test pH increased by >1 unit in all flasks where algal growth was observed. This is attributed to algal growth and did not have an impact on the outcome of the study (Section 4.2.5).

The temperature recorded continuously during the definitive test was in the range 22.7-23.8 °C.

4.2.3 *Statistical Analysis*

The 72 h EC_{50} value for WRS-2390TX, based on the area under the curve (AUC), was determined to be 0.06 mg/L.

The 72 h EC_{50} value for WRS-2390TX, based on growth rate, was determined to be 0.13 mg/L.

The No Observed Effect Concentration (NOEC) at 72h was 0.02 mg/L, based on AUC and <0.02 mg/L based on growth rate.

The 96 h EC_{50} value for WRS-2390TX, based on the area under the curve (AUC), was determined to be 0.07 mg/L.

The 96 h EC_{50} value for WRS-2390TX, based on growth rate, was determined to be 0.16 mg/L.

The No Observed Effect Concentration (NOEC) at 96h was <0.02 mg/L, based on AUC and 0.11 mg/L based on growth rate.

The EC_{50} value for AUC at 72 h and Growth Rate at 24, 48, and 72 h are lower than the 96 h values. This is attributed to the growth pattern observed during the test as illustrated in Figures 2 and 3.

A report detailing the statistical procedures used is presented in Appendix 3.

4.2.4 Re-Inoculation Phase

Based on statistical analysis of the cell numbers observed after the 9 day re-inoculation phase no significant differences were found in growth rate. This indicated that the effect of the test item to algal cells was phytostatic and not phytotoxic. However, the cells in the test item treated flasks were noted to have formed clumps whereas growth in the control flasks was normal.

4.2.5 Validity of the Test

The cell density of control cultures increased by a factor of *ca* 140 which greatly exceeded the ≥ 16 required in the protocol. The pH of the control cultures in 5 of the 6 replicates increased by up to 2.5 pH units over the 96 h period. However, based on the growth data, this was not thought to have affected the outcome of the study.

The coefficient of variation in daily growth in the control cultures was < 35% in line with protocol requirements (Appendix 3, Table 3). The coefficient of variation of average growth exceeded 15 % (Appendix 3, Table 4) in all control replicates, which is a deviation from the protocol. However, Figure 2 indicated that logarithmic growth was achieved in control cultures after a 24 h lag period, in line with the OPPTS Guideline. Hence this study is reported and the results regarded as acceptable.

5 Conclusion

For the green alga *Pseudokirchneriella subcapitata*, the 96 hour EC_{50} value of WRS-2390TX for area under the growth curve was calculated to be 0.07 mg/L (measured).

The 96 hour EC_{50} value of WRS-2390TX based on growth rate was calculated to be 0.16 mg/L (measured).

The No Observed Effect Concentration at 96 h based on AUC was < 0.02 mg/L.

The No Observed Effect Concentration at 96 h based on growth rate was 0.11 mg/L.

The effect of the test item to algal cells was phytostatic and not phytotoxic, as no significant differences were found in growth rate after 9 days in fresh untreated medium. However the cells in the two test item treatments were both noted to have formed clumps, compared to the controls where growth was normal.

6 Protocol Deviations

The culture used to inoculate the test flasks was sub-cultured for 1 day in test conditions rather than 3-5 days following initiation from a refrigerated slope and cultured for 3 days. However growth in the sub-culture appeared sufficient to allow use at test initiation. A lag phase was observed in the first 24 h of the test, however from 48 h, growth appeared to be logarithmic so the impact on the test was minimal.

Certain validity criteria were not achieved however these are discussed in Section 4.2.5, and the study is regarded as suitable to report.

7 References

OECD (2002) Guidelines for the Testing of Chemicals. Proposal for Updating Guideline 201, 'Freshwater Alga and Cyanobacteria, Growth Inhibition Test'.

US EPA (1996) Public Draft: Ecological Effects Test Guidelines , OPPTS 850.5400 Algal Toxicity, Tiers I and II, EPA 712-C-96-164.

8 Tables

Table 1 ***Mean Cell Concentration (x 10^5 cell/mL) and Percentage Growth Inhibition During the Range Finding Test***

Time (h)	Replicate / Mean	Nominal Concentration of WRS-2390TX (mg/L)				
		Control	0.001	0.01	0.1	1.0
0	Mean	0.1	0.1	0.1	0.1	0.1
72	I	8.65	7.03	8.58	6.50	0.03
	II	8.28	7.08	7.48	7.68	0.08
	Mean	8.47	7.06	8.03	7.09	0.06
% Inhibition Compared to Control		NA	16.6	5.2	16.3	99.3

NA = Not Applicable

Table 2 ***Mean Measured Concentrations of WRS-2390TX at 0 h and 96 h During the Definitive Test***

Nominal Concentration (mg/L)[1]	Mean Measured Concentration (mg/L)				Geometric Mean	% of Nominal
	0 h	% of Nominal	96 h	% of Nominal		
Control	ND	ND	ND	ND	NA	NA
0.03	0.03	100	0.01*	33	0.02	67
0.06	0.06	100	0.02* 0.05**	33 83*	0.03	50
0.14	0.14	100	0.09	64	0.11	79
0.31	0.32	103	0.22	71	0.27	87
0.67	0.67	100	0.56 0.51**	84 76*	0.61	91

ND = Not Detectable
NA = Not Applicable

[1]= Values corrected for purity (71.2%)

* = Concentrations lower than the lowest analytical standard prepared (0.03 mg/L) and calculated by linear extrapolation
** = Mean measured concentrations and % of nominal concentrations in samples containing no algae

Table 3 ***Mean Cell Concentrations (x 10^5 cell/mL) During the Definitive Test***

Time (h)	Replicate / Mean	Geometric Mean Measured Concentration of WRS-2390TX (mg/L)					
		Control	0.02	0.03	0.11	0.27	0.61
0	Mean	0.1	0.1	0.1	0.1	0.1	0.1
24	I	0.25	0.43	0.35	0.15	0.15	0.08
	II	0.30	0.18	0.28	0.10	0.03	0.03
	III	0.33	0.28	0.08	0.20	0.13	0.08
	IV	0.38	-	-	-	-	-
	V	0.18	-	-	-	-	-
	VI	0.30	-	-	-	-	-
	Mean	0.29	0.30	0.24	0.15	0.10	0.06
48	I	1.30	1.55	1.60	0.48	0.33	0.05
	II	1.90	1.00	1.25	0.50	0.20	0.05
	III	1.80	1.95	1.30	0.60	0.20	0.03
	IV	1.55	-	-	-	-	-
	V	1.50	-	-	-	-	-
	VI	1.85	-	-	-	-	-
	Mean	1.65	1.50	1.38	0.53	0.24	0.04
72	I	6.65	5.10	5.90	2.83	0.20	0.05
	II	7.05	4.93	4.00	4.65	0.18	0.05
	III	6.25	4.60	3.30	2.95	0.03	0.05
	IV	6.40	-	-	-	-	-
	V	5.85	-	-	-	-	-
	VI	5.33	-	-	-	-	-
	Mean	6.26	4.88	4.40	3.48	0.14	0.05
96	I	20.55	9.90	12.80	7.98	0.50	0.03
	II	13.50	12.10	11.35	9.60	0.10	0.13
	III	12.80	10.83	10.35	7.40	0.20	0.05
	IV	14.05	-	-	-	-	-
	V	10.90	-	-	-	-	-
	VI	12.55	-	-	-	-	-
	Mean	14.06	10.94	11.50	8.33	0.27	0.07

- = Not Applicable

Table 4 ***Biomass Values (AUC) for all Replicates and Treatment Group Means for the Definitive Test***

Geometric Mean Measured Concentration of WRS-2390TX (mg/L)	Replicate	Time (h)			
		0-24	0-48	0-72	0-96
Control	1	7500	37500	154167	453125
	2	10000	55000	182500	391250
	3	11500	54000	166833	360750
	4	13000	49250	162000	374625
	5	4000	39000	145167	315750
	6	10000	53750	152167	335125
	Mean	9333	48083	160472	371771
0.02	1	16500	52750	142667	292000
	2	4000	26500	113167	295250
	3	9000	55250	142667	297375
	Mean	9833	44833	132834	294875
0.03	1	12500	50000	155000	347500
	2	9000	37750	109333	271375
	3	0	30000	93333	238125
	Mean	7167	39250	119222	285667
0.11	1	2500	12000	59833	177500
	2	0	10000	89167	242500
	3	5000	17500	67500	177500
	Mean	2500	13167	72167	199167
0.27	1	2500	8250	11000	14500
	2	0	2500	4667	4500
	3	1500	4000	4333	4500
	Mean	1333	4917	6667	7833
0.61	1	0	0	0	0
	2	0	0	0	375
	3	0	0	0	0
	Mean	0	0	0	125

Table 5 ***Growth Rate Values per Hour for all Replicates and Treatment Group Means for the Definitive Test***

Geometric Mean Measured Concentration of WRS-2390TX (mg/L)	Replicate	Time (h)			
		0-24	0-48	0-72	0-96
Control	1	0.038	0.053	0.058	0.055
	2	0.046	0.061	0.059	0.051
	3	0.050	0.060	0.057	0.050
	4	0.053	0.057	0.058	0.052
	5	0.024	0.056	0.057	0.049
	6	0.046	0.061	0.055	0.050
	Mean	0.043	0.058	0.058	0.051
0.02	1	0.061	0.057	0.055	0.048
	2	0.024	0.048	0.054	0.050
	3	0.043	0.062	0.053	0.049
	Mean	0.043	0.056	0.054	0.049
0.03	1	0.052	0.058	0.057	0.051
	2	0.043	0.053	0.051	0.049
	3	0	0.053	0.049	0.048
	Mean	0.032	0.055	0.052	0.050
0.11	1	0.017	0.033	0.046	0.046
	2	0	0.034	0.053	0.048
	3	0.029	0.037	0.047	0.045
	Mean	0.015	0.035	0.049	0.046
0.27	1	0.017	0.025	0.010	0.017
	2	0	0.014	0.008	0
	3	0.011	0.014	0	0.007
	Mean	0.009	0.018	0.006	0.008
0.61	1	0	0	0	0
	2	0	0	0	0.027
	3	0	0	0	0
	Mean	0	0	0	0.009

Means from unrounded data.

Table 6 ***pH Values at 0 h and 96 h During the Definitive Test***

Time	Replicate	Geometric Mean Measured Concentration of WRS-2390TX (mg/L)					
		Control	0.02	0.03	0.11	0.27	0.61
0 h	Stock	7.6	7.8	7.7	7.7	7.7	7.7
96 h	I	7.81	10.01	9.99	9.57	7.88	7.78
	II	10.05	10.06	9.95	9.54	7.81	7.75
	III	10.08	10.00	10.00	10.04	7.78	7.78
	IV	10.08	-	7.98*	-	-	7.82*
	V	10.10	-	-	-	-	-
	VI	10.06	-	-	-	-	-

- = Not Applicable

* = Uninoculated flask

9 Figures

Figure 1 ***Mean Area Under Curve (0-96 h)***

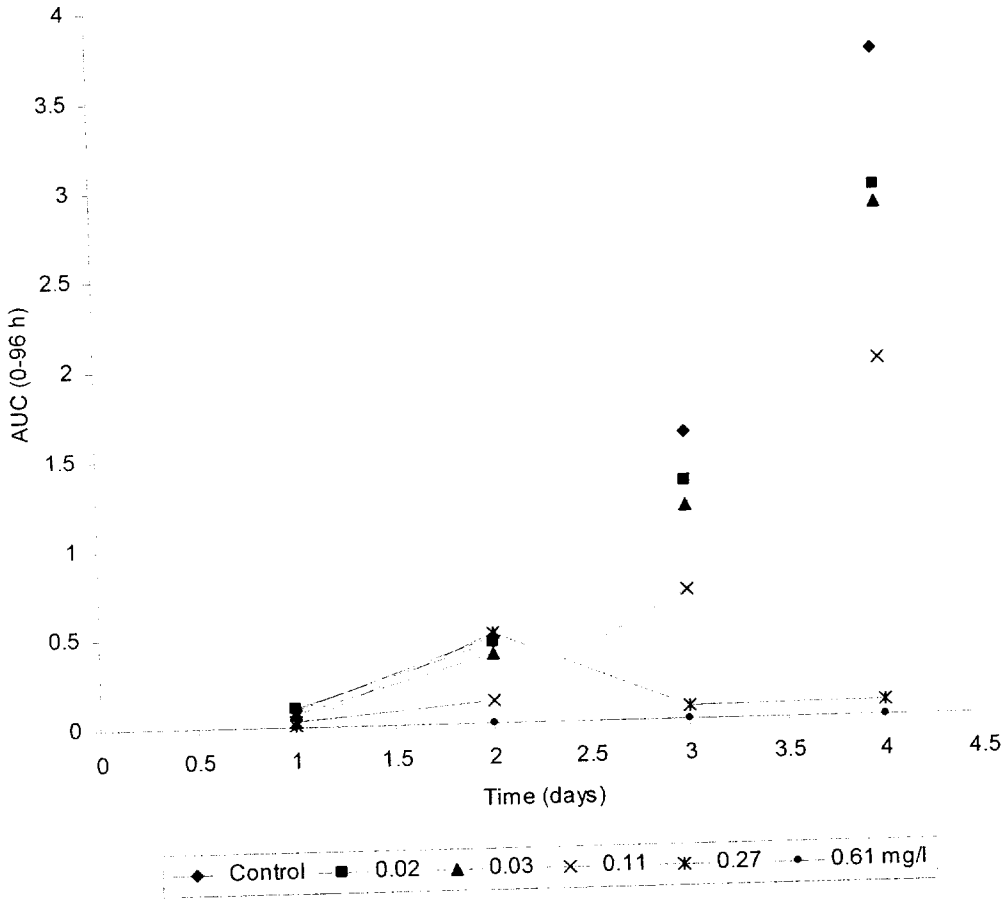


Figure 2 ***Mean Inhibition of Area Under Curve Over 96 h***

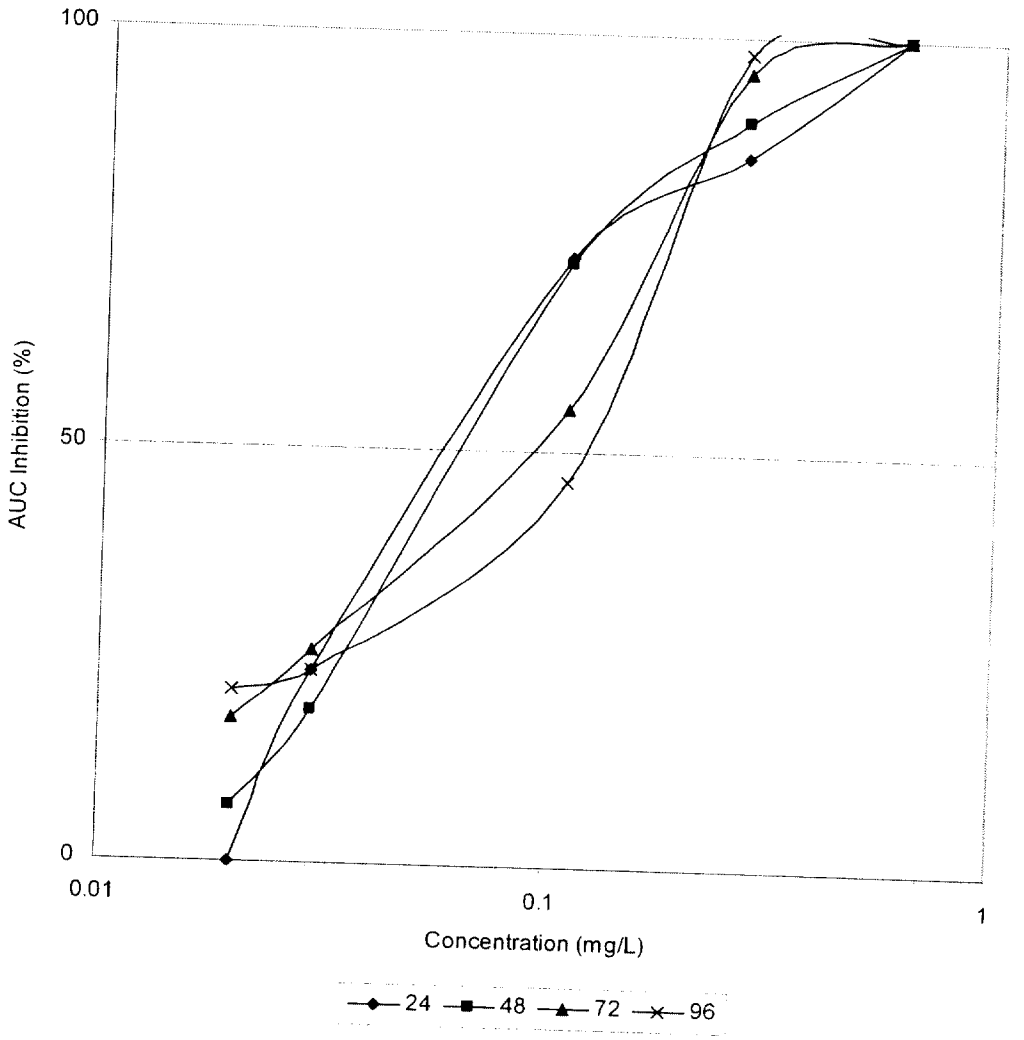


Figure 3 ***Mean Inhibition of Growth Rate Over 96 h***

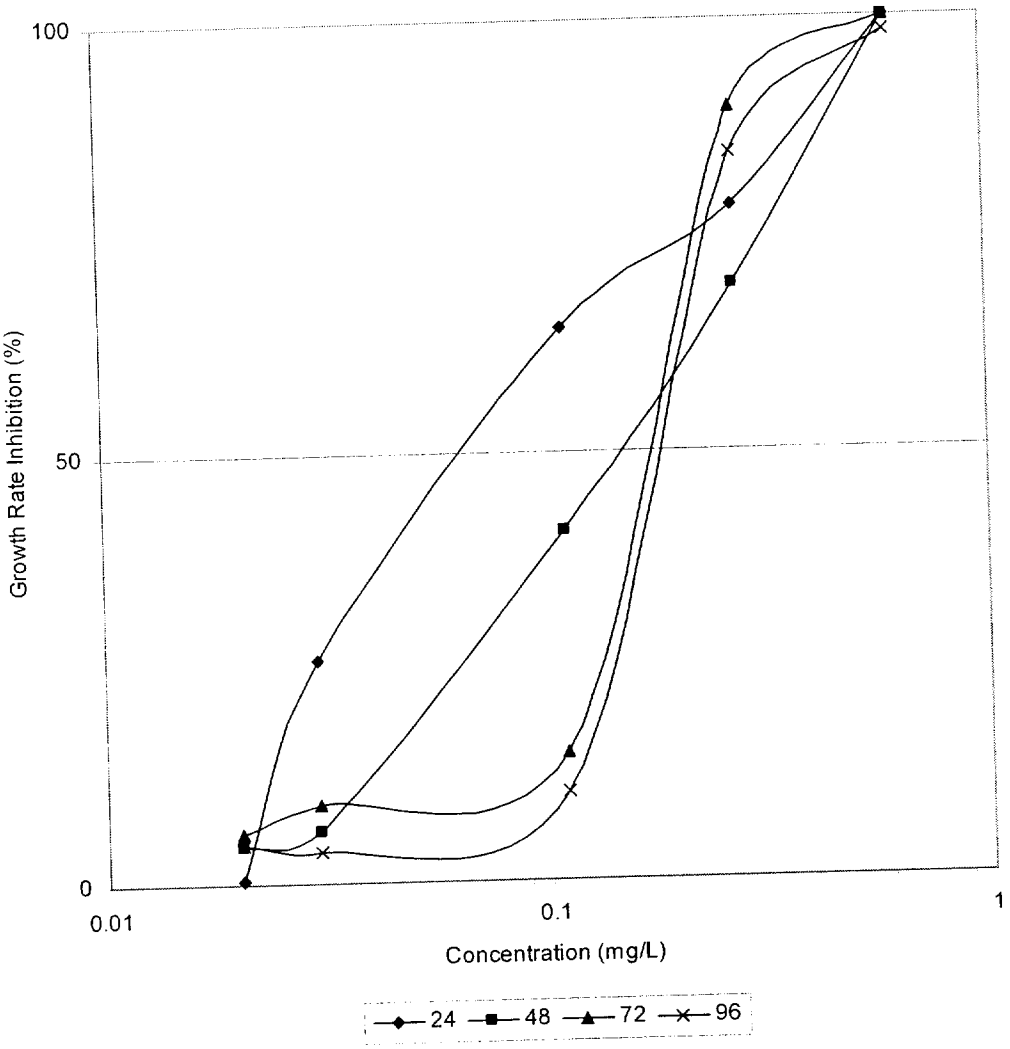
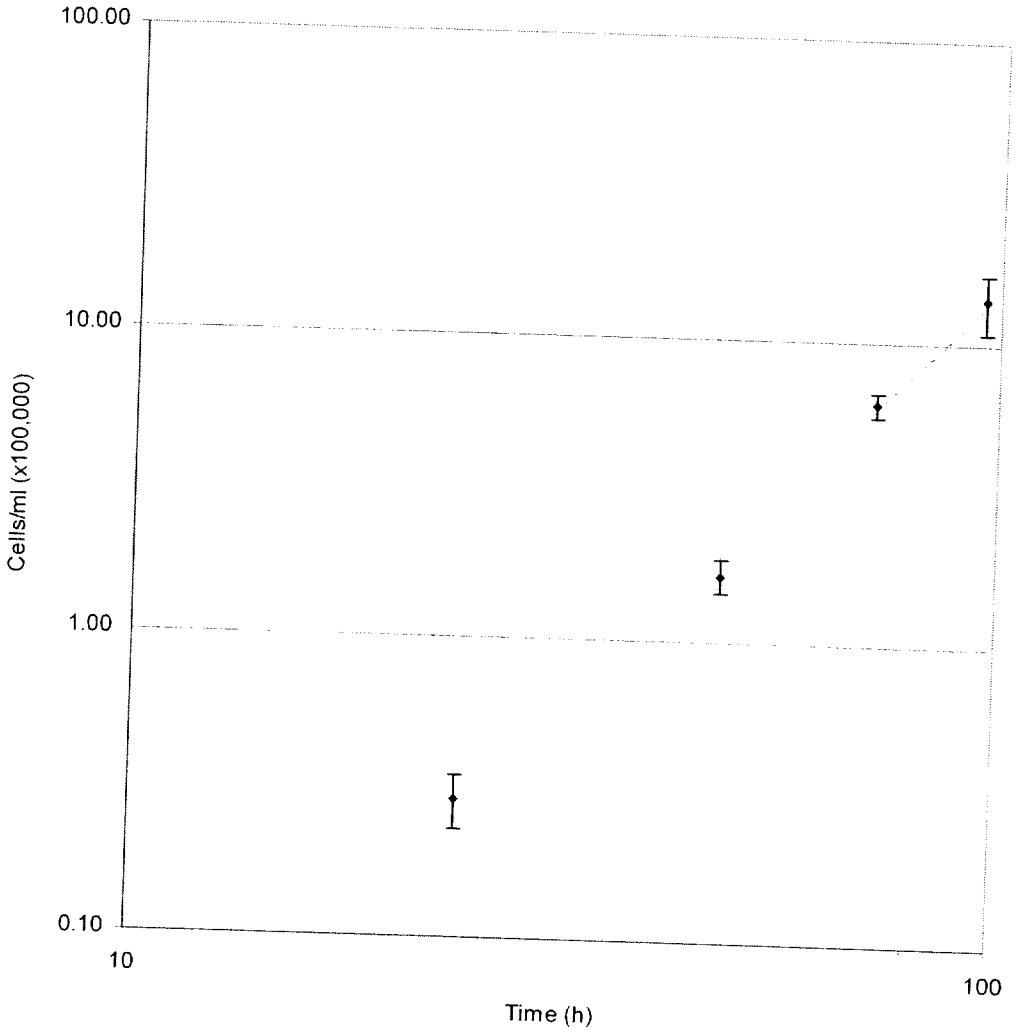


Figure 4 ***Mean Cell Density in Control Cultures***



10 Appendices

Appendix 1 Preparation of Algal Growth Medium

ISO Freshwater Algal Growth Medium was prepared by addition of weighed quantities of analytical grade chemicals to de-ionised water, to give the following concentrations:

Constituent	Concentration (mg/L)
NH_4Cl	15
$MgCl_2.6H_2O$	12
$CaCl_2.2H_2O$	18
$MgSO_4.7H_2O$	15
KH_2PO_4	1.6
$FeCl_3.6H_2O$	0.08
$Na_2EDTA.2H_2O$	0.1
H_3BO_3	0.185
$MnCl_2.4H_2O$	0.415
$ZnCl_2$	3×10^{-3}
$COCl_2.6H_2O$	1.5×10^{-3}
$CuCl_2.2H_2O$	1×10^{-5}
$NaMoO_4.2H_2O$	7×10^{-3}
$NaHCO_3$	50

Appendix 2 Analytical Method No. 4275

8 Appendix

Appendix 1 Analytical Method No. 4275

Assay: HPLC (Range 1.42 mg.l^{-1} to 0.036 mg.l^{-1})	Analytical Method No.: 4275
Test Item: WRS-2390	Date of Approval: 05 December 2003
	Approved: A Hogg

I. Summary

Solutions of WRS-2390 are assayed by high performance liquid chromatography. Detection is by uv and external standardisation is used. Other instrumentation may be used providing adequate accuracy and precision can be achieved.

II. Reagents

a) WRS-2390 (purity 71.2 %).
b) Phosphoric acid.
c) Milli Q water or equivalent grade.
d) Acetonitrile, HPLC grade.
e) Milli RO water or equivalent.

III. Apparatus

a) LC Module 1, supplied by Waters or equivalent.
b) Balance, 5 figure.
c) Laboratory glassware.
d) Volumetric glassware.
e) Sonic bath.

IV. Chromatographic Conditions/Set Up

HPLC: Waters LC Module 1.
Column: YMC ODS-AM, 150 mm x 4.6 mm, 3 µm.
Column temperature: 40°C.
Mobile phase A: 0.1 % phosphoric acid in Milli Q water.
Mobile phase B: acetonitrile.
Detection: uv at 225 nm.
Injection volume: 150 µl.
Run time: 20 min.

Appendix 2 (continued) *Analytical Method No. 4275*

Appendix 1 (continued)

Flow rate: 1 $ml.min^{-1}$.
Needle wash: Milli Q water/acetonitrile (9:1, v/v).
Data collection: LabSystems Vax Multichrom 2 version 2.30b or other suitable data collection system.
Gradient timetable:

Time (min)	% Mobile Phase A	% Mobile Phase B	Curve
0	90	10	-
1	90	10	6
10	5	95	6
12	5	95	6
12.10	90	10	6
20	90	10	6

V. Preparation of Standard Curve

Accurately weigh *ca* 50 mg of WRS-2390 into a 100 ml volumetric flask then dissolve and make to volume using acetonitrile to give a *ca* 500 $mg.l^{-1}$ solution. Sonicate to aid dissolution. Dilute the *ca* 500 $mg.l^{-1}$ solution by adding 2.5 ml to a 50 ml volumetric flask and make to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) to give a *ca* 25 $mg.l^{-1}$ solution. Dilute this solution as detailed in the following table:

Standard Identity	Concentration of Stock Solution used ($mg.l^{-1}$)	Volume Diluted (ml)	Final Volume (ml)	*Nominal Concentration ($mg.l^{-1}$)
B1	25	4	50	1.42
B2	25	3	50	1.07
B3	25	2	50	0.71
B4	25	1	50	0.36
B5	25	1	100	0.18
B6	2	5	100	0.071
B7	2	2.5	100	0.036

* corrected for test item purity (71.2 %)

Make each standard to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v).

Note: Alternative dilutions may be performed providing the sample final concentration is achieved.

Analyse each standard solution, in duplicate, according to the chromatographic conditions detailed in Section IV.

Note: For analysis of study samples, the lowest standard prepared was 0.03 mg/L

Appendix 2 (continued) Analytical Method No. 4275

Appendix 1 (continued)

VI. Preparation of Quality Control Sample

Accurately weigh *ca* 50 mg of WRS-2390 into a 100 ml volumetric flask then dissolve and make to volume using acetonitrile to give a *ca* 500 $mg.l^{-1}$ solution. Sonicate to aid dissolution. Dilute the *ca* 500 $mg.l^{-1}$ solution by adding 2.5 ml to a 50 ml volumetric flask and make to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) to give a *ca* 25 $mg.l^{-1}$ solution. Dilute this solution by adding 2 ml to a 50 ml volumetric flask and make to volume using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) to give a *ca* 1 $mg.l^{-1}$ (0.71 $mg.l^{-1}$ purity corrected) solution.

Note: Alternative dilutions may be performed providing the sample final concentration is achieved.

Analyse the *ca* 1 $mg.l^{-1}$ (0.71 $mg.l^{-1}$ purity corrected) solution, in duplicate, according to the chromatographic conditions detailed in Section IV.

VII. Preparation of Aqueous Samples for Analysis

Aqueous solutions of WRS-2390 in Milli RO water or algae or daphnia media may be directly injected onto the analytical column and analysed according to the chromatographic conditions detailed in Section IV, providing the sample concentration is within the range of the standard curve detailed in Section V.

If required, aqueous solutions of WRS-2390 in Milli RO water or algae or daphnia media may be diluted to within the range of the standard curve detailed in Section V using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v).

Note: The method validation indicated that direct injections of WRS-2390 in aqueous media resulted in *ca* 82 % recoveries from the nominal applied concentration. If possible, aqueous samples should be diluted using 0.1% phosphoric acid in Milli Q water/acetonitrile (9:1, v/v) prior to analysis. Otherwise the accuracy of the results should be quoted as 100 % ± 20 % of the found concentration.

The diluted samples should be analysed, in duplicate, according to the chromatographic conditions detailed in Section IV.

Appendix 3 Statistical Analysis

STATISTICAL REPORT

Date: 18 May 2004

To: Mary Murphy

From: Sharon Laing

Subject: ***804607***

1. Statistical Methods

The main objectives of this study were to calculate the no observed effect concentration (NOEC) and the EC_{50} value with respect to cell concentrations measured in algae.

Cell concentrations measured at 0, 24, 48, 72 and 96 hours for control and 5 active concentrations of WRS-2390 TX were available for statistical analysis. The geometric means of the measured concentrations of the actives were: 0.02, 0.03, 0.11, 0.27 and 0.61 mg/L. Any cell concentrations at 24, 48, 72 and 96 hours less than the measured cell concentration at 0 h were replaced by the overall mean cell concentration at 0 h. The measured cell concentration at 0 h was 10000 cell/mL.

In this study two active concentrations and the control underwent re-inoculation. The geometric means of the measured concentrations of the actives were: 0.27 and 0.61 mg/L. Re-inoculation and incubation at the test conditions was for 9 days. Cell concentrations measured at 9 days were available for statistical analysis with the initial 0 days cell concentrations for the re-inoculation phase taken as a 1 in twenty dilution of the 96 h cell counts for each replicate.

Following the OECD guideline, normalised Area Under the double linear growth Curves (AUC) were calculated for the purposes of statistical analysis, over the following time intervals:

0-24 hours post dose inclusive (AUC 0-24 h)
0-48 hours post dose inclusive (AUC 0-48 h)
0-72 hours post dose inclusive (AUC 0-72 h)
0-96 hours post dose inclusive (AUC 0-96 h)

Normalised AUC values were obtained by dividing calculated AUC values by the number of hours in the particular interval. Furthermore, the growth rate values were calculated over the same time intervals.

The AUC and growth rate values were analysed separately for homogeneity of variance using Levene's test[1] at a 1% significance level. If there was no evidence of heterogeneity of variance, the AUC and growth rate values were analysed using

Appendix 3 (continued) *Statistical Analysis*

analysis of variance (ANOVA) techniques[2]. Following the ANOVA, pairwise comparisons were performed between the control and mean measured concentrations using a one-tailed Dunnett's test[3] at the 5% significance level.

If there was evidence of heterogeneity of variance, the AUC and growth rate values were transformed using a log transformation. The transformed values were then re-analysed separately for homogeneity of variance using Levene's test[1] at a 1% significance level. If there was no evidence of heterogeneity of variance, the transformed AUC and growth rate values were to be analysed in an identical way to the untransformed values.

If the variability between concentrations was still heterogeneous after having tried the log transformation then a square root transformation was performed.

If the variability between concentrations was still heterogeneous after having tried the square root transformation then a non-parametric analysis of variance based on the ranked data was performed [4]. Following the non-parametric ANOVA, pairwise comparisons between the control and each of the mean measured concentrations were performed using a one-tailed Dunnett's test[3] at the 5% significance level.

Based on the assumption of a linear dose-response relationship, the NOEC is defined to be the highest concentration which is not significantly different from the control at a 5% significance level using a one-tailed Dunnett's test.

The EC_{50} value is defined to be the concentration causing a 50% decrease in mean cells with respect to the above parameters. The % inhibition values were calculated using the mean value for control and the mean value for each concentration of WRS-2390 TX. A probit transformation was applied to the % inhibition values for each parameter separately. The probit transformed data were subjected to a regression procedure against logarithmically transformed concentrations of test material with a Newton-Raphson maximum likelihood iterative procedure being used to obtain parameter estimates[5]. From the fitted model, the EC_{50} value was estimated.

The goodness of fit of the probit model to the AUC and growth rate data was checked via the Pearson chi-squared test statistic. A significant chi-squared test statistic indicated heterogeneity between the observed and expected % inhibition values. If the test statistic was statistically significant at the 1% level, the variances and covariances were adjusted by a heterogeneity factor. Following this, the confidence limits were not reported and the EC_{50} value should be treated with caution.

For the re-inoculation period, growth rates were calculated from the start of the re-inoculation period, and were analysed using ANOVA techniques[2]. Following the ANOVA, pairwise comparisons were performed between the control and mean measured concentrations using a one-tailed Dunnett's test[3] at the 5% significance level.

Homogeneity of variance was assessed using Levene's test at a 1% significance level. If there had been evidence of heterogeneity of variance, then log and square

Appendix 3 (continued) *Statistical Analysis*

root transformations would have been explored, as described above. If there had still been evidence of heterogeneity of variance, then a non-parametric analysis of variance based on the ranked data would have been performed.

Additionally, the coefficient of variation was calculated for replicates of each control and test treatment for both daily and average growth.

Appendix 3 (continued) *Statistical Analysis*

2. Results

The results of the statistical analyses are presented in Tables 1-3 below.

TABLE 1

Area under the Growth Curve and Growth Rate: EC_{50} and NOEC values

Parameter	EC_{50} (mg/L)		NOEC
	Estimate	95% CI	(mg/L)
AUC(0-24h)	0.08	-	0.11
AUC(0-48h)	0.07	0.06 – 0.08	0.03
AUC(0-72h)	0.06	-	0.02
AUC(0-96h)	0.07	-	< 0.02
GROWTH RATE(0-24h)	0.08	-	0.03
GROWTH RATE(0-48h)	0.13	0.11 – 0.15	0.03
GROWTH RATE(0-72h)	0.13	-	< 0.02
GROWTH RATE(0-96h)	0.16	-	0.11

When calculating the EC_{50} values the Pearson chi-square goodness of fit test statistic was statistically significant at the 1% level for all parameters, except AUC (0-48h) and Growth Rate (0-48h), and as a result no confidence intervals have been reported and the EC_{50} values should be treated with caution.

When calculating the NOEC values the assumption of homogeneity of variance was satisfied for all parameters with the exception of AUC (0-48h) and Growth Rate (0-72h), and as a result, the NOEC for all parameters with the exception of AUC (0-48h) and Growth Rate (0-72h) was obtained using a parametric analysis without the use of a transformation, and followed by Dunnetts test. For AUC (0-48h) the homogeneity of variance was satisfied for square root transformed data. For Growth Rate (0-72h), the homogeneity of variance was not satisfied for either untransformed, or square root transformed data, and as a result the NOEC was obtained using a non-parametric ANOVA, followed by Dunnett's test.

Appendix 3 (continued) *Statistical Analysis*

TABLE 2

Coefficient of Variation in Daily Growth Rates

Geometric Mean of Measured Concentration (mg/L)	CV% GROWTH RATE(0-24h)	CV% GROWTH RATE(24-48h)	CV% GROWTH RATE(48-72h)	CV% GROWTH RATE(72-96h)
0.00	24.10	12.59	14.23	23.37
0.02	42.47	20.33	30.39	15.53
0.03	87.84	32.81	16.63	19.31
0.11	95.09	21.56	17.60	17.62
0.27	92.37	29.06	-69.20	238.34
0.61	*	*	*	173.21

* Could not be calculated as growth rates for these time periods were zero.

TABLE 3

Coefficient of Variation of Average Growth in Replicates

Geometric Mean of Measured Concentration (mg/L)	CV% Replicate 1	CV% Replicate 2	CV% Replicate 3	CV% Replicate 4	CV% Replicate 5	CV% Replicate 6
0.00	27.59	40.50	33.02	25.04	61.85	34.84
0.02	29.79	45.36	44.35			
0.03	25.92	18.36	91.40			
0.11	51.26	85.87	35.53			
0.27	159.07	*	348.68			
0.61	*	200.00	*			

* Could not be calculated as growth rates for these concentrations were zero.

For the re-inoculation period the assumption of homogeneity of variance was satisfied for Growth Rate (0-9 days), and as a result, parametric analysis of the data was conducted without the use of a transformation, and followed by Dunnetts test. Using this method it was found that there were no significant difference between the active concentrations and the control at the 5% significance level.

A copy of all QC checks performed on data entry and all statistical analysis performed on these data are included.

Appendix 3 (continued) *Statistical Analysis*

3. References

(1) Levene H (1960)
("Robust tests for equality of variances")
"Contributions to Probability and Statistics - Essays in Honour of Harold Hotelling"
Olkin et al (eds)
Stanford University Press
Stanford
California
p278-292

(2) Snedecor G.W. and Cochran W.G. (1980)
Statistical Methods (7th edition)
The Iowa State University Press
Ames, Iowa, USA, p215-237

(3) Dunnett C.W. (1964)
"New Tables for Multiple Comparisons with a Control"
Biometerics 20, p482-491

(4) Conover W. J. and Iman R. L. (1981)
Rank Transformations as a Bridge between Parametric and Non-parametric Statistics.
The American Statistician 35, p124-128

(5) Finney D. J. (1971)
Probit Analysis
Third Edition
Cambridge University Press
University Printing House, Cambridge

Appendix 4 GLP Certificate

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY

Inveresk Research International Ltd.
Elphinstone Research Centre
Tranent
East Lothian
EH33 2NE

Including

Veterinary Clinical Trials Unit
Talkin
Brampton
Cumbria
CA8 1LE

TEST TYPE

Analytical Chemistry
Clinical Chemistry
Ecosystems
Environmental Toxicity
Environmental Fate
Mutagenicity
Phys/chem Tests
Toxicology

Safety studies on Veterinary products

DATE OF INSPECTION

13 April 2004

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Bryan Wright

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority